

Gene cluster conservation provides insight into cercosporin biosynthesis and extends production to the genus *Colletotrichum*

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Species in the genus *Cercospora* cause economically devastating diseases in sugar beet, maize, rice, soy bean, and other major food crops. Here, we sequenced the genome of the sugar beet pathogen *Cercospora beticola* and found it encodes 63 putative secondary metabolite gene clusters, including the cercosporin toxin biosynthesis (*CTB*) cluster. We show that the *CTB* gene cluster has experienced multiple duplications and horizontal transfers across a spectrum of plant pathogenic fungi, including the wide-host range *Colletotrichum* genus as well as the rice pathogen *Magnaporthe oryzae*. Although cercosporin biosynthesis has been thought to rely on an eight-gene *CTB* cluster, our phylogenomic analysis revealed gene collinearity adjacent to the established cluster in all *CTB* cluster-harboring species. We demonstrate that the *CTB* cluster is larger than previously recognized and includes cercosporin facilitator protein, previously shown to be involved with cercosporin autoresistance, and four additional genes required for cercosporin biosynthesis, including the final pathway enzymes that install the unusual cercosporin methylenedioxy bridge. Lastly, we demonstrate production of cercosporin by *Colletotrichum fioriniae*, the first known cercosporin producer within this agriculturally important genus. Thus, our results provide insight into the intricate evolution and biology of a toxin critical to agriculture and broaden the production of cercosporin to another fungal genus containing many plant pathogens of important crops worldwide.

natural product | perylenequinone | secondary metabolism | cercosporin | *Cercospora*

Among the most speciose genera in all of Fungi are the *Cercospora* (1). First described in 1863 (2), the genus has sustained a long history, largely due to notoriety as the causal agent of leaf spot diseases in a wide range of plants, including agriculturally important crops such as sugar beet, soybean, maize, and rice that together account for hundreds of millions of dollars in lost revenue annually to growers worldwide (3–8). Although *Cercospora* spp. share several characteristics associated with pathogenicity, such as penetration through natural openings and extracellular growth during the biotrophic stage of infection, most rely on the production of the secondary metabolite (SM) cercosporin (1) to facilitate infection (9, 10). Studies spanning nearly 60 y have made cercosporin a model perylenequinone (11), a class of SMs characterized by a core pentacyclic conjugated chromophore that gives rise to its photoactivity. When exposed to ambient light, cercosporin is a potent producer of reactive oxygen species in the presence of oxygen (12), with a quantum efficiency of >80% (13). This small molecule is lipophilic and can readily penetrate plant leaves, leading to indiscriminate cellular damage within minutes of exposure (14).

Indeed, cercosporin is nearly universally toxic to a wide array of organisms, including bacteria, mammals, plants, and most fungal species, with the key exception of cercosporin-producing fungi, which exhibit cercosporin autoresistance. To date, cercosporin has been reported to be produced only by *Cercospora* spp., with the single exception of the brassica pathogen *Pseudocercospora capsellae* (15). However, *Pseudocercospora* and *Cercospora* are phylogenetically closely related, residing in a large clade within Mycosphaerellaceae (16).

In contrast to the large body of information on cercosporin biology spanning several decades (17, 18), the cercosporin toxin

Significance

Species in the fungal genus *Cercospora* cause diseases in many important crops worldwide. Their success as pathogens is largely due to the secretion of cercosporin during infection. We report that the cercosporin toxin biosynthesis (*CTB*) gene cluster is ancient and was horizontally transferred to diverse fungal plant pathogens. Because our analyses revealed genes adjacent to the established *CTB* cluster with similar evolutionary trajectories, we evaluated their role in *Cercospora beticola* to show that four are necessary for cercosporin biosynthesis. Lastly, we confirmed that the apple pathogen *Colletotrichum fioriniae* produces cercosporin, the first case outside the family Mycosphaerellaceae. Other *Colletotrichum* plant pathogens also harbor the *CTB* cluster, which points to a wider role that this toxin may play in virulence.

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biosynthesis (*CTB*) gene cluster was only recently resolved in *Cercospora nicotianae* (19). The keystone enzyme for cercosporin biosynthesis, *CTB1*, bears all the hallmarks of an iterative, nonreducing polyketide synthase (PKS) (20). Using *CTB1* as a point of reference, the complete *C. nicotianae* *CTB* gene cluster was determined to consist of eight contiguous genes, of which six are believed to be responsible for cercosporin assembly (*CTB1*, 2, 3, 5, 6, and 7) (19, 21). The zinc finger transcription factor *CTB8* coregulates expression of the cluster (19), while the major facilitator superfamily transporter *CTB4* exports the final metabolite (22). Downstream of the *CTB* cluster are two ORFs encoding truncated transcription factors, while loci designated as *ORF9* and *ORF10* upstream of the *CTB* cluster are not regulated by light and are not believed to encode proteins with metabolic functions (19). Consequently, the clustering of eight genes with demonstrated coregulation by light that are flanked by ORFs with no apparent role in cercosporin biosynthesis has suggested that cercosporin production relies on the eight-gene *CTB* cluster (19). In this study, we used an evolutionary comparative genomics approach to show that the *CTB* gene cluster underwent multiple duplication events and was transferred horizontally across large taxonomic distances. Since these horizontal transfer events included genes adjacent to the canonical eight-gene *CTB* cluster, we used reverse genetics to show that the *CTB* cluster includes additional genes in *Cercospora beticola*, including one gene that was previously shown to be involved with cercosporin autoresistance (23) and four previously unrecognized genes involved with biosynthesis. The *CTB* cluster was found in several *Colletotrichum* spp., and we confirmed that the apple pathogen *Colletotrichum fioriniae* can also produce cercosporin. As all earlier understanding of cercosporin biosynthesis has been unwittingly limited by a truncated set of genes in *Cercospora* spp., the full dimension of the gene cluster provides deeper insight into the evolution, biosynthesis, and dissemination of a fungal toxin critical to worldwide agriculture.

Results

SM Cluster Expansion in *C. beticola*. *C. beticola* strain 09-40 was sequenced to 100-fold coverage and scaffolded with optical and genome maps, resulting in 96.5% of the 37.06-Mbp assembly being placed in 12 supercontigs, of which 10 are assumed to be chromosomes. Despite their ubiquitous presence in nature and cropping systems, genome sequences of *Cercospora* spp. are not well represented in public databases. Therefore, to aid comparative analysis within the *Cercospora* genus, we also sequenced the genome of *Cercospora berteroae* and reassembled the genome of *Cercospora canescens* (24) (SI Appendix, Table S1). To identify gene clusters responsible for biosynthesis of aromatic polyketides in *C. beticola*, we mined the genome to identify all SM clusters (25) and compared these with predicted clusters in related Dothideomycetes. The *C. beticola* genome possesses a total of 63 predicted SM clusters of several classes, representing an expanded SM repertoire with almost twice the number as compared with closely related Dothideomycetes fungi, which average 34 SM clusters (SI Appendix, Table S2 and Dataset S1). Notably, *C. beticola* encodes 23 candidate nonribosomal peptide synthetase clusters, which is considerably higher than most Dothideomycetes fungi, which have an average of 13 (26). To identify the *C. beticola* PKS cluster responsible for cercosporin biosynthesis, we compared the sequence of the *C. nicotianae* *CTB* cluster (19) with predicted PKS clusters of *C. beticola*. To fill in sequencing gaps between genes in the *C. nicotianae* *CTB* cluster, we sequenced the genome of *C. nicotianae*, which showed that *C. beticola* PKS *CBET3_00833* (*CbCTB1*) and flanking genes (*CBET3_00830* – *CBET3_00837*) were ~96% identical to *C. nicotianae* *CTB1* to *CTB8*, and all genes were collinear, strongly suggesting that this region houses the *CTB* cluster in *C. beticola* (SI Appendix, Fig. S1).

Repeated Duplication and Lateral Transfer of the Cercosporin Biosynthetic Cluster. To study the evolutionary relationships of *C. beticola* PKSs, we conducted large-scale phylogenomic analyses that included various previously characterized PKSs from selected species (27). Because resolving orthologous relationships among PKSs can predict the type of SM that will be synthesized, we first built a phylogenetic tree of the conserved core β -ketoacyl synthase domains of each PKS that resulted in separating PKS enzymes into four major groups (SI Appendix, Fig. S2A). Among the eight *C. beticola* nonreducing PKSs, phylogenetic analysis revealed significant similarity among *CbCTB1*, *CBET3_10910-RA*, and *CBET3_11350-RA*, which cluster at the base of the cercosporin clade (SI Appendix, Fig. S2B). Interestingly, genes flanking *CBET3_10910-RA*, but not *CBET3_11350-RA*, were also strikingly similar to *CbCTB* cluster genes (Fig. 1). Consequently, we hypothesized that the *CBET3_10910* SM cluster is the result of a *CTB* cluster duplication. Since duplicated SM gene clusters appeared to be relatively rare in fungi (28), we investigated the origin and specificity of the *CTB* cluster and the putative duplication by searching for *CbCTB1* homologs against a selected set of 48 published Ascomycetes proteomes (SI Appendix, Table S3) representing a diverse group of fungal orders. We identified *CbCTB1* orthologs in the *Cercospora* spp. *C. berteroae* and *C. canescens* and confirmed its presence in *Cladosporium fulvum* (27) and *Parastagonospora nodorum* (29). Surprisingly, seven additional orthologs were identified in Sordariomycetes species (*Colletotrichum orbiculare*, *Colletotrichum gloeosporioides*, *C. fioriniae*, *Colletotrichum graminicola*, *Colletotrichum higginsianum*, and *Magnaporthe oryzae*), as well as one within Leotiomycetes (*Sclerotinia sclerotiorum*) (SI Appendix, Fig. S3A), representing diverse taxa harboring *CTB1*. Analysis of sequence identity showed that intraspecies (*CbCTB1* to *CBET3_10910-RA*) sequence identity (45%) was lower than the interspecies [e.g., *CbCTB1* and *C. fulvum* *CTB1* (Clafu1_196875)] sequence identity (55%) (SI Appendix, Table S4), suggesting that the *CTB1* duplication event occurred before Dothideomycetes speciation.

To develop a “phylogenetic roadmap” that may explain *CTB1* evolution, we used the process of “reconciliation” that takes into account both species and gene histories (30). Although not conclusive, reconciliation considers the costs of evolutionary events (i.e., gene duplications, transfers, and/or losses) to explain the most parsimonious evolutionary route to the present scenario (31). Reconciliation of the species tree (SI Appendix, Fig. S4) with the *CTB1* protein tree revealed that the predicted evolutionary history of *CTB1* can be characterized by four duplications, three

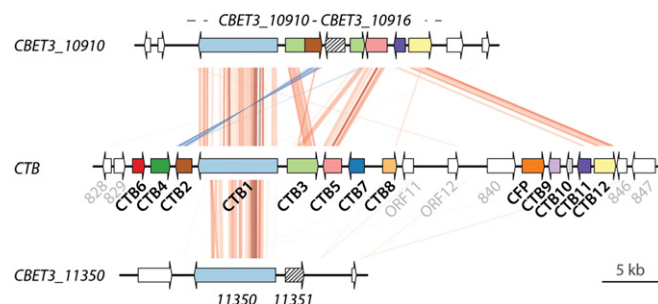


Fig. 1. The cercosporin biosynthetic cluster is duplicated and maintained in *C. beticola*. *CBET3_10910* and flanking genes are syntenic with the *CTB* cluster (*CBET3_00833* and flanking genes) in *C. beticola*. Alignment lines correspond to DNA fragments exhibiting significant similarity when the genomic regions comprising the gene clusters are compared with tBLASTx. Direct hits are displayed in red, whereas complementary hits are in blue. The intensity of the alignments represents the percentage similarity ranging from 23 to 100%. Genes flanking *CBET3_11350-RA* were not syntenic with *CTB* cluster genes.

transfers, and widespread loss to most species analyzed (*SI Appendix*, Fig. S5A) and further corroborates our hypothesis that the *CTB1* duplication event (D1) occurred early in Dothideomycetes speciation. Reconciliation also revealed an ancient *CTB1* ortholog in *S. sclerotiorum* (*SI Appendix*, Fig. S5A), suggesting that *CTB1* arose before speciation of Dothideomycetes. Duplications D2 to D4 arose after lateral transfer (T1) of *CTB1* into the last common ancestor of Glomerellales. *CTB1* was then transferred (T2) from a common ancestor in Glomerellales to *M. oryzae* (*SI Appendix*, Fig. S5A).

We extended the search for CTB cluster protein orthologs by scanning the 48 proteomes for homologs of *CbCTB2* (CBET3_00830) to *CbCTB8* (CBET3_00837), followed by phylogenetic tree construction and subtree selection (*SI Appendix*, Fig. S3 B–N). This resulted in the identification of orthologs in the same set of species previously listed to contain *CTB1*, with the only exceptions in cases where *CTB* gene homologs were lost in a species. Although the loss of *CTB6* and *CTB7* orthologs limits reconciliation analysis of these gene families, reconciliation of the subtrees for *CTB2*, *CTB3*, *CTB4*, *CTB5*, and *CTB8* (*SI Appendix*, Fig. S5 B–H) supported a similar scenario as proposed for *CTB1*, involving at least two duplications (D1 and D2) and two horizontal transfer events (T1 and T2) that explain the present-day *CTB* scenario (Fig. 2). However, an alternative explanation involving a single transfer to an ancestral Glomerellales species followed by widespread loss in most species in this lineage, except for *M. oryzae* and the analyzed *Colletotrichum* spp. (Fig. 2 and *SI Appendix*, Table S5), cannot be ruled out by our analyses at this stage.

Extension of the Predicted Cercosporin Biosynthetic Cluster Based on Microsynteny. To further examine the *CTB* clusters across all recipient species, we generated pairwise alignments relative to the *C. beticola* *CTB* cluster and flanks. To our surprise, we observed a striking level of similarity outside the known eight *CTB* genes on the 3' end of the cluster (Fig. 3) in all *CTB*-containing genomes. To investigate whether the amount of microsynteny observed for the *CTB* cluster and these flanking genes can be reasonably expected when comparing Dothideomycetes and Sordariomycetes genomes, we assessed the genome-wide microsynteny between the genomes of *C. beticola* and *C. gloeosporioides* and between *C. beticola* and *M. oryzae*. This analysis identified the *CTB* cluster together with its flanking genes as having the highest level of microsynteny among all regions in the genome between *C. beticola* and *C. gloeosporioides* and showed that the observed *CTB* microsynteny between *C. beticola* and *M. oryzae* was also higher than the genome-wide average (Fig. 4). Likewise, sequence identity of most *CTB* proteins between *C. beticola* and *Colletotrichum* spp., and to a lesser degree with *M. oryzae*, is higher compared with the genome-wide average (*SI Appendix*, Figs. S6 and S7). In contrast, sequence conservation of *CTB8*, a Zn₂Cys₆ transcription factor previously implicated for transcriptional regulation of the *CTB* cluster (19), appears much lower than that of other *CTB* and non-*CTB* proteins and, therefore, is suggestive of positive diversifying selection. Considering the level of microsynteny and protein conservation, we hypothesized that these flanking genes are part of the *C. beticola* *CTB* cluster. To test this proposal, we first determined the relative expression of all eight established *C. beticola* *CTB* genes as well as a number of flanking genes (CBET3_00828 to CBET3_00848) under light (cercosporin-inducing) vs. dark (cercosporin-repressing) conditions, which showed that all candidate *CTB* genes on the 3' flank were induced in the light, except CBET3_00846 and CBET3_00848 (*SI Appendix*, Table S7). Functional annotation of these genes revealed one nonconserved phenylalanine ammonia lyase (CBET3_00840), the cercosporin facilitator protein (CFP) (23) (CBET3_00841), a candidate α -ketoglutarate-dependent dioxygenase (CBET3_00842), a dehydratase (CBET3_00843), a

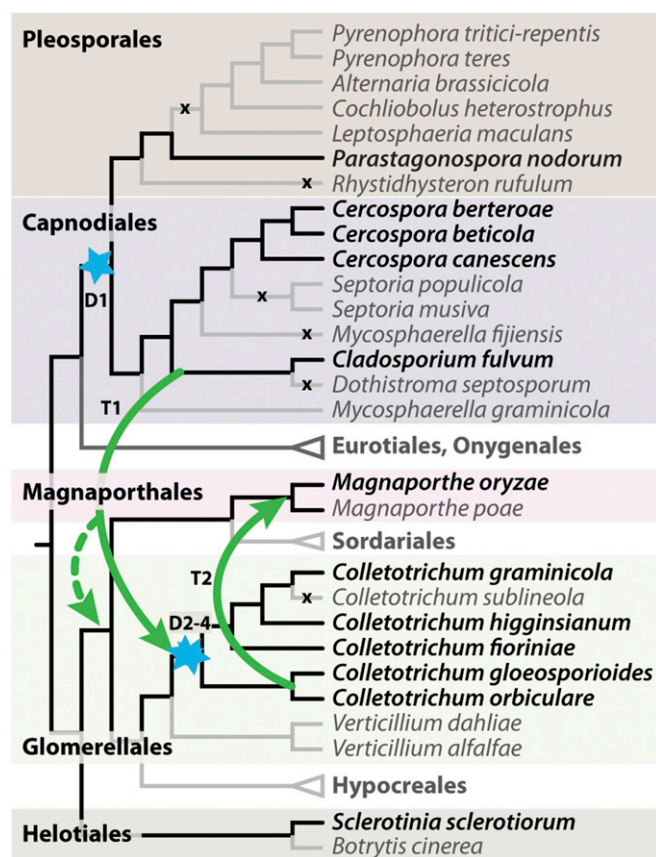


Fig. 2. Phylogenetic roadmap of *CTB* cluster evolution. Phylogenetic roadmap detailing the proposed evolutionary trajectory of the *CTB* cluster involving horizontal gene transfer events from Capnodiales to Glomerellales (T1) and another from Glomerellales to Magnaporthales (T2), as well as multiple duplications (D1 to D4) and frequent gene loss (x). Cladogram of the phylogenetic relationship of *Cercospora* spp. and 45 other sequenced fungi. The unscaled tree was constructed using CTVTree. Duplication nodes are marked with blue stars, losses are indicated by x's, and transfers are highlighted by green arrows. Species without the *CTB* cluster are depicted in gray; those encompassing it are in black. An alternative and slightly less parsimonious scenario involving a single transfer from Capnodiales into the last common ancestor of Magnaporthales and Glomerellales is shown by the dashed arrow.

β -ig-h3 fasciclin (CBET3_00844), a laccase (CBET3_00845), zinc finger domain-containing protein (CBET3_00846), and protein phosphatase 2A (CBET3_00847; *SI Appendix*, Table S7), several of which have functions associated with multidomain enzymes or polyketide biosynthesis in fungi or bacteria (19, 22, 32–36). Phylogenetic analyses of these flanking genes and reconciliation of their respective protein phylogenies (*SI Appendix*, Figs. S3 and S5) with the species tree (*SI Appendix*, Fig. S4) suggest that all genes except CBET3_00840, CBET3_00846, and CBET3_00847 have undergone highly similar evolutionary trajectories as the established *CTB* cluster genes (Fig. 2 and *SI Appendix*, Fig. S5), suggesting that the *CTB* cluster was transferred as a whole at least once, followed by species-specific evolutionary trajectories involving frequent gene loss as well as gene gain (Fig. 2). We further evaluated the hypothesis of horizontal cluster transfer using a comparative topology test that examines whether the determined tree topologies that support horizontal cluster transfer are significantly better than constrained topologies that would not support transfer. Tree topologies were compared using the Approximately Unbiased test (37), implemented in CONSEL (38) as previously described by Wisecaver and Rokas (39).

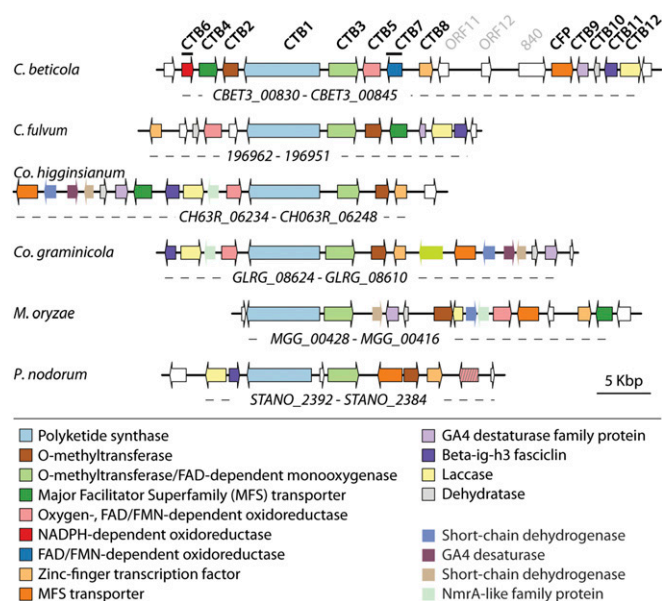


Fig. 3. Synteny and rearrangements of the conserved *C. beticola* cercosporin biosynthetic cluster. The cercosporin biosynthetic cluster in *C. beticola* (top line) and flanking genes are conserved in *C. fulvum*, *C. higginsianum*, *C. graminicola*, *M. oryzae*, and *P. nodorum*. For all species, the displayed identifiers are transcript IDs, and the corresponding sequences can be retrieved from JGI MycoCosm or ORCAE. *CTB* orthologs are colored relative to the *C. beticola* *CTB* cluster genes; the color key and annotated functions are highlighted below the *CTB* cluster graphic. *Cercospora*-specific *CTB* genes *CTB6* and *CTB7* are underlined.

Constrained topologies in which we force either a monophyletic origin of all Dothideomycetes proteins or a monophyletic origin of all Sordariomycetes proteins were significantly worse than trees without such constraint (SI Appendix, Table S6). Thus, the comparative topology tests support the previously determined topologies, which suggest horizontal cluster transfer.

CTB Genes Essential for Cercosporin Biosynthesis. To confirm individual gene contributions for cercosporin production, we generated single-gene deletion mutants of all candidate genes from *CBET3_00840* to *CBET3_00846* and tested their ability to produce cercosporin. Initial assays of selected mutants showed that cercosporin production in $\Delta CBET3_00844$ and $\Delta CBET3_00845$ mutants was abolished, while $\Delta CBET3_00842$ mutants accumulated only a red, cercosporin-like metabolite that migrated differently in potato dextrose agar (PDA) culture plates and TLC (SI Appendix, Fig. S8). To provide more definitive analyses of cercosporin production, HPLC profiles were obtained from all candidate *CTB* gene mutants and compared with WT cercosporin (Fig. 5A). Unlike other analyzed mutants, $\Delta CBET3_00840$ and $\Delta CBET3_00846$ produced compounds with HPLC profiles like cercosporin (Fig. 5A), suggesting that these genes are not involved with cercosporin biosynthesis. Together, these results corroborate our hypothesis that the *CTB* cluster extends to at least *CBET3_00845* at the 3' side and includes four additional *CTB* biosynthetic genes, as well as *CbCFP*. Consequently, we propose naming genes *CBET3_00842*, *CBET3_00843*, *CBET3_00844*, and *CBET3_00845* as *CTB9* to *CTB12*, respectively (SI Appendix, Table S7).

Precercosporin Isolation and Characterization. To characterize the red metabolite that accumulated in the $\Delta 842/CTB9$ and $\Delta 843/CTB10$ mutants (Fig. 5A and SI Appendix, Fig. S8), an ethyl acetate extract of the collected mycelia was analyzed by reverse-phase HPLC. At 280 nm, a single peak was observed in both

mutant extracts with identical retention times and UV-Vis spectra (Fig. 5). This peak was compared with a reference sample of cercosporin produced by wild-type *C. beticola*. The retention time of this peak was shorter than that of cercosporin, suggesting a more polar metabolite. Comparison of the UV-Vis spectra (Fig. 5B–D) of the unknown compound and cercosporin revealed nearly identical chromophores, suggesting close structural relation. The exact mass of the metabolite from the mutants was determined ($\Delta 842/CTB9$: m/z 537.1762, $\Delta 843/CTB10$: m/z 537.1757, $[M+H]^+$), consistent with the elemental composition $C_{29}H_{28}O_{10}$. This mass is 2 Da greater than that of cercosporin (+2 hydrogens), which led to a proposed structure for precercosporin (2) (Fig. 6). Alternative hydroquinones of cercosporin could be excluded simply on the basis of the UV-Vis spectral information and chemical instability. The presence of a free phenol in precercosporin in place of the unusual seven-membered methylenedioxy of cercosporin is consonant with the red shift of the long wavelength (λ_{max}) and the shorter HPLC retention time.

To firmly support the tentative structure of precercosporin, the crude extract of $\Delta 842/CTB9$ was further purified by reverse-phase HPLC. To obtain sufficient material for 1H -NMR analysis, extractions were performed quickly and in low light and reduced temperature to slow apparent polymerization of precercosporin. The relative instability of precercosporin compared with cercosporin suggests a possible role for the methylenedioxy bridge in overall stability. Immediately evident from the 1H -NMR spectrum (SI Appendix, Fig. S9A), apart from its overall similarity to that of cercosporin itself, was not only the absence of the methylenedioxy singlet at δ 5.74 diagnostic of cercosporin but also the appearance of a new methoxyl signal at δ 4.28 and a phenol at δ 9.25. Consistent with the new asymmetry in precercosporin, two strongly hydrogen-bonded *peri*-hydroxy groups could be seen far downfield at around 15 ppm, and two aryl hydrogens were observed at δ 6.92 and δ 6.87. That these latter resonances are observed only in pairs (as are the two side-chain methyl doublets at around 0.6 ppm) and the other signals are doubled implies that precercosporin is formed as a single atropisomer having a helical configuration likely identical to that of cercosporin, although it is conceivable that *CTB9* or *CTB10* sets the final stereochemistry.

^{13}C -NMR spectroscopy data were obtained by growing a larger number of PDA plates of $\Delta 842/CTB9$ supplemented with 2 mM $[1-^{13}C]$ -sodium acetate and 2 mM $[2-^{13}C]$ -sodium acetate to equally enrich all polyketide-derived carbons (~3% per site). Working quickly to isolate and purify precercosporin in low light and low temperature as described earlier, both 1D and heteronuclear single quantum coherence spectra of precercosporin were acquired (SI Appendix, Fig. S9B and C). As seen in the 1H -NMR spectrum, breaking the symmetry of cercosporin was evident in the observation of all 29 carbons in the ^{13}C -NMR

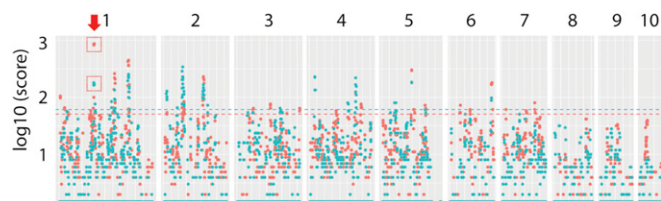


Fig. 4. *CTB* cluster microsynteny conservation segregates from the genome-wide average. The genome-wide, gene-by-gene microsynteny between *C. beticola* and *C. gloeosporioides* (depicted in red) and between *C. beticola* and *M. oryzae* (in blue) across the 10 assembled *C. beticola* chromosomes is shown. Each dot represents one *C. beticola* gene and its respective microsynteny score. The red arrow indicates the position of the *CTB* cluster on chromosome 1 and coincides with high microsynteny in both *C. gloeosporioides* and *M. oryzae*. The dashed lines represent the 99th quantile of the microsynteny scores for both comparisons independently.

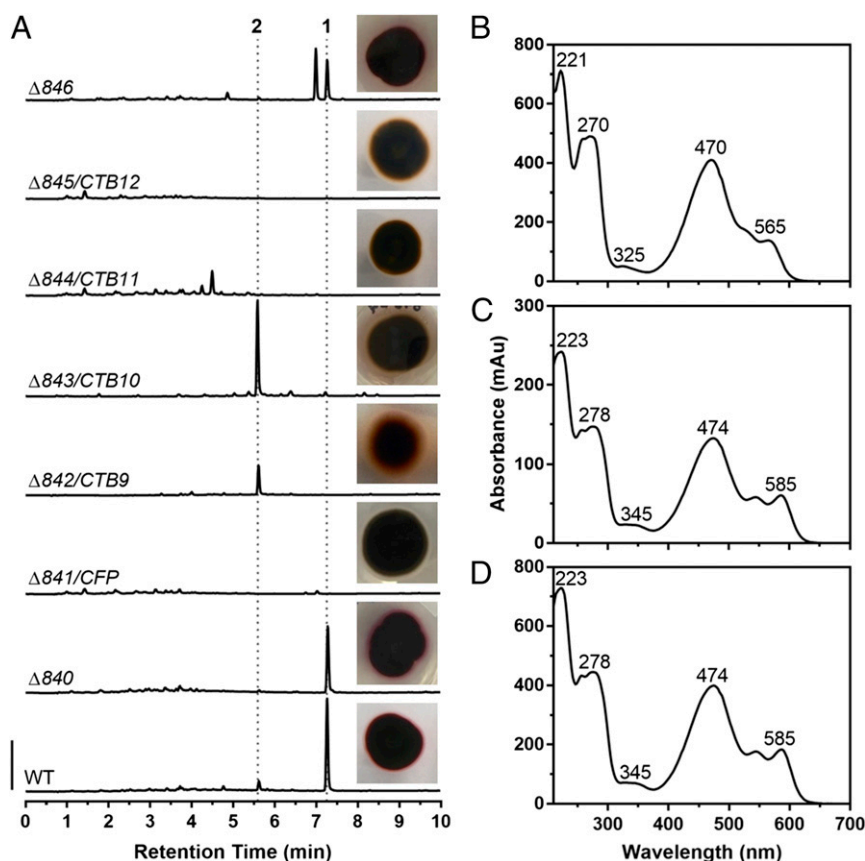


Fig. 5. Analysis of cercosporin production in *CTB* mutants of *C. beticola*. Site-directed knockout mutants in genes *CBET3_00840*, *CFP* (*CBET3_00841*), *CTB9* (*CBET3_00842*), *CTB10* (*CBET3_00843*), *CTB11* (*CBET3_00844*), *CTB12* (*CBET3_00845*), and *CBET3_00846* were assayed for cercosporin production by HPLC. Cercosporin extracted from *C. beticola* strain 10-73-4 (WT) was used as a positive control. (A) 280 nm HPLC chromatograms and images of representative colonies for each knockout. (Scale bar, 250 mAu.) Cercosporin (1) and precercosporin (2) peaks are indicated by dashed lines. (B–D) UV-Vis spectra from wild-type *C. beticola* (7.25-min peak in B), *C. beticola* Δ CTB9 (5.36-min peak in C), and *C. beticola* Δ CTB10 (5.36-min peak in D) were extracted from 280 nm HPLC chromatograms. Wavelengths of relevant UV maxima are indicated.

spectrum, which notably revealed three methoxyl groups and diagnostic doubling of all resonances, save two overlapping pairs of signals. This behavior is fully in accord with the assigned structure of precercosporin.

Identification of Cercosporin from *C. fioriniae*. Because our initial phylogenomic analyses suggested that several *Colletotrichum* spp. harbored *CTB* clusters (Figs. 2 and 3), we questioned whether the *CTB* cluster can be found in additional *Colletotrichum* spp. *CTB* protein orthology analysis revealed that 8 of the 13 *Colletotrichum* spp. hosted at Ensembl Fungi (<https://fungi.ensembl.org/index.html>) encode a similar set of *CTB* proteins as observed in *C. higginsianum* (SI Appendix, Table S8). These eight species are plant pathogens of crops such as apple, safflower, melon, and cucumber; a variety of *Brassica* and cereal crops; as well as various tree species (40–46) (SI Appendix, Figs. S10 and S11 and Table S8). Remarkably, many species have lost several *CTB* genes, such as the endophyte *Colletotrichum tofieldiae*, which has lost the cluster entirely (SI Appendix, Figs. S10 and S11 and Table S8).

Earlier reports suggested the production of a red pigment by some *Colletotrichum* spp. such as the apple pathogen *C. fioriniae* (47, 48); therefore, we questioned whether the red pigment was cercosporin. As a first step, two *C. fioriniae* strains (HC89 and HC91) from our collection that were previously isolated from apple were assayed for cercosporin production using the KOH assay (49). No cercosporinlike pigment was observed in the

medium under the same conditions that stimulate cercosporin production in *C. beticola*. Since epigenetic modifiers have been used to induce production of SMs in fungal species (50, 51), we considered whether this strategy could be used to induce cercosporin production in *C. fioriniae*. Medium augmented with the histone deacetylase inhibitor trichostatin A (TSA) (50) induced

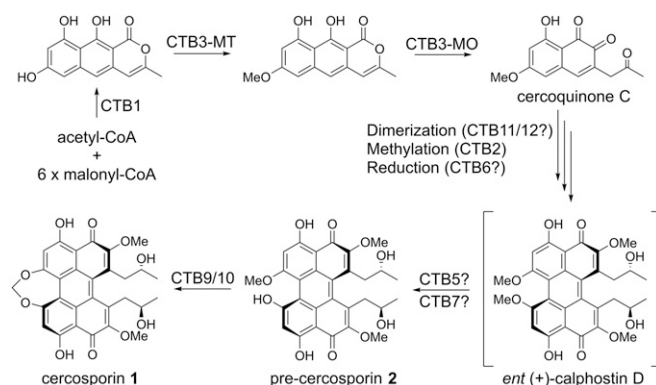


Fig. 6. Proposed biogenesis of cercosporin. Tentative proposal for biosynthesis of cercosporin (1), incorporating biosynthetic genes identified in this study. Intermediates in brackets are logically inferred and have not been directly observed. MO, monooxygenase; MT, methyltransferase.

cultivars will be necessary to confirm whether cercosporin is necessary for virulence of this pathogen.

The microsynteny outside the established *CTB* cluster prompted us to test whether the flanking genes in *C. beticola* are also required for cercosporin biosynthesis. Notably, we observed that these flanking genes, similar to the established *CTB* genes, were up-regulated under cercosporin-inducing conditions. Furthermore, targeted gene replacement of *CTB9*, *CTB10*, *CTB11*, and *CTB12* completely abolished cercosporin biosynthesis, while replacement of *CTB9* and *CTB10* resulted in the accumulation of a red metabolite, defined here as precercosporin. We thus conclude that the *CTB* cluster is significantly larger than previously described (19).

The isolation and characterization of an intermediate in the cercosporin biosynthetic pathway (precercosporin) strongly suggests that formation of the unique seven-membered methylenedioxy bridge in the final product is the result of a two-step process requiring three genes. First, one of two precursor aryl methoxyl groups of *ent* (+)-calphostin D (Fig. 6) is removed, followed by oxidative ring closure by *CTB9*, an apparent α -ketoglutarate-dependent dioxygenase, in collaboration with *CTB10*. The precise role of *CTB10*, a putative dehydratase, in ring closure is unclear, but it could serve to facilitate closure of the unfavorable seven-membered methylenedioxy ring. In contrast, a single cytochrome P450 is known to convert two aryl *ortho*-methoxyl groups into the relatively more common five-membered methylenedioxy group in alkaloid biosynthesis (61). We attribute the single demethylation to an oxidative process possibly carried out by the flavin-dependent enzymes *CTB5* or *CTB7*. *CTB6* correlates to the SDR NAD(P)H-binding superfamily of oxidoreductases and could install the side-chain hydroxyl groups stereospecifically. Owing to the extreme instability of most pathway intermediates and the role feedback inhibition in response to these metabolites could play, our experience dictates that analysis of pathway knockouts alone will not lead to the full determination of cercosporin biosynthesis. Biochemical evaluation of the individual enzymes, as has been done with *CTB3* (21) with synthetic substrates and product standards, will be necessary to accomplish this task.

A tentative cercosporin biosynthesis scheme was recently proposed (21) without knowledge of the expanded *CTB* cluster. However, in light of the identification of precercosporin and the potential functions of the other *CTB* genes, the previously proposed biosynthetic pathway (21) will have to be revised. While these investigations will be reported in due course, we suspect the fasciclin/laccase pair (*CTB11/12*) may act early in the pathway to dimerize the product of *CTB3* (21) to the first perylenequinone intermediate, which would have precedent in synthetic chemistry (62) and in simpler laccase-mediated aryl-aryl dimerizations (35, 63, 64) (Fig. 6). *CTB1* is an iterative, nonreducing PKS whose product is *nor*-toralactone (20). *CTB3* is a bifunctional enzyme (an *O*-methyltransferase and FAD-dependent monooxygenase) that carries out sequential *O*-methylation in the presence of *S*-adenosylmethionine and oxidative decarboxylation to cercoquinone C (21). We hypothesize that intermediate steps of *O*-dimethylation and side-chain ketone reduction, in unspecified order, are mediated by *CTB2* and potentially *CTB6*, respectively, as noted above.

Despite sustained research on cercosporin for several decades, there are significant knowledge gaps in cercosporin biosynthesis.

Our data have shed light on cercosporin biology and will have significant impact on cercosporin research in particular and on perylenequinone research in general. The finding that at least one species in the important plant pathogenic genus *Colletotrichum* can produce cercosporin has significant implications for disease management. Moreover, since *C. fioriniae* may secrete cercosporin into apple food products that may be directly consumed by humans, the toxic effects of cercosporin on human health may need to be considered.

Materials and Methods

For further information, see [SI Appendix, SI Materials and Methods](#) and figshare under DOI: 10.6084/m9.figshare.4056522. Custom code is permanently archived at Zenodo under DOI: 10.5281/zenodo.1156551.

Fungal genomic DNA was isolated from mycelia scraped from the surface of agar Petri plates. Library preparations and sequencing on the Illumina platform was performed by BGI Americas Corp. For *C. beticola*, three genomic libraries with increasing insert size (500 bp, 5 Kbp, and 10 Kbp) were sequenced. For *C. berteroae*, *C. nicotianae*, and *C. fioriniae* strains HC89 and HC91, single, short insert libraries (500 bp) were sequenced. For *C. beticola* specifically, optical maps were prepared using the Argus (OpGen) and BioNano Genomics platforms and subsequently used to scaffold contigs into large supercontigs. A combination of *ab initio* gene prediction, homologous protein alignment, and transcript alignment followed by extensive manual curation was used to prepare draft gene models for *C. beticola*. *C. beticola*-trained Augustus parameters were used for automated protein-coding gene modeling in the case of *C. berteroae*, *C. canescens*, and *C. nicotianae*. Genome assemblies and annotations, if applicable, are deposited in the NCBI GenBank database and listed under BioProject PRJA270309. Accession numbers for *C. beticola*, *C. berteroae*, *C. nicotianae*, and *C. fioriniae* strains HC89 and HC91 are LKMD000000000, PNEN000000000, POSS000000000, and PNFI000000000, respectively.

Mycelial plugs of wild-type and mutant *C. beticola* were placed on top of eight "thin" PDA (Difco) plates (3.0 mL of PDA per 50-mm Petri plate). Cultures were incubated at 22 °C for 1 wk under continuous light. PDA and mycelia were ground under liquid nitrogen and lyophilized to dryness twice. The resulting powder was resuspended in water acidified with HCl (pH <1), allowed to sit 10 min, and filtered. The filtrate was extracted thrice with ethyl acetate. These extracts were pooled, washed with brine, and evaporated to dryness. The extracted metabolites were resuspended in 500 μ L of methanol and analyzed by HPLC on an Agilent 1200 fitted with a Kinetex XB-C18 column (4.6 \times 75 mm, 2.6 μ m; Phenomenex). Injections of 1 μ L were run at 1.25 mL/min with a linear gradient of 5% A/95% B to 95% A/5% B over 10.8 min, where solvent A was acetonitrile + 0.1% formic acid and solvent B was 0.1% formic acid. Chromatograms were monitored at 436, 280, and 210 nm, and UV-Vis spectra were recorded over a range of 210 to 800 nm. High-resolution mass data were obtained from a Waters Acquity/ Xevo-G2 UPLC-ESI-MS in positive-ion mode.

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